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Brief Report

Adeno-Associated Virus Gene Transfer to Mouse Retina

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ABSTRACT

Ocular gene transfer may provide a means for arresting the retinal degeneration characteristic of many inherited causes of blindness, including retinitis pigmentosa (RP). Previously, we have shown in immunodeficient animals that recombinant adeno-associated virus (rAAV) mediates transduction of photoreceptors as well as the retinal pigment epithelium (RPE) following subretinal injection. In this study we extend these observations and show that highly purified recombinant AAV vectors encoding the reporter gene *LacZ* transduce photoreceptors in an immunocompetent mouse strain following subretinal injection and efficiently transduce ganglion cells after intravitreal injection. Levels of transduction increase over time. Sublethal γ-irradiation is shown to facilitate this process.

INTRODUCTION

RETINITIS PIGMENTOSA is one of the most common inherited eye disorders, affecting approximately 1 in 3,500 of the general population and causing progressive blindness. These diseases are characterized by peripheral retinal degeneration with loss of rods and subsequently cone cells leading to loss of central vision. At least 30 mapped loci have been directly associated with RP. Seven different nonsyndromic RP genes have been identified to date, five of which are expressed exclusively in photoreceptor cells (Dryja *et al.*, 1990, 1995; Farrar *et al.*, 1991; McLaughlin *et al.*, 1993; Bascom *et al.*, 1995). At present, treatment that will retard or prevent the onset of blindness is not available. However, transfer of a normal copy of the defective gene to these cells may arrest the degenerative process and preserve vision to a degree relating to maturity of the disease process. The photoreceptor layer appears to be most accessible to gene transfer by subretinal injection. However, experience obtained with adenovirus-based delivery systems suggests that transduction of photoreceptors is relatively inefficient, particularly in adult animals (Bennett *et al.*, 1994; Li *et al.*, 1994; Mashour *et al.*, 1994). An alternative treatment strategy for RP might be to delay photoreceptor degeneration by increasing the intraocular levels of neurotrophic factors. This strategy does not rely on transduction of neuroretina. Secreted neurotrophic factors could be produced by any transduced cell. The number of transduced retinal pigment epithelium (RPE) cells following subretinal injection of adenovirus and the number of transduced cells in the anterior segment (ciliary body, iris, trabecular meshwork, and corneal endothelium) following intravitreal injection is much greater than the number of cells transduced in the neuroretina (Ali *et al.*, 1997). Recent data suggest that adenovirus-mediated expression of ciliary neurotrophic factor, after intravitreal injection of virus, is able to delay retinal degeneration in the *rd* mouse, an animal model of RP (Cayouette and Gravel, 1997). The effectiveness of this approach, however, may be limited by the toxicity of adenovirus at high multiplicities of infection (Li *et al.*, 1994), and by immune responses directed against viral genes expressed from the vector backbone (manuscript in preparation).

Adeno-associated virus (AAV) is a nonpathogenic human parvovirus that has attracted considerable interest as a gene transfer vector (Kotin, 1994). Replication of this virus is usually dependent on co-infection with a helper virus. In the ab-

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sence of helper virus (usually adenovirus) the wild-type AAV genome can integrate stably into the host cell genome by nonhomologous recombination, usually in a tandem head-to-tail orientation. Analysis of flanking sequences from latently infected human cells has shown that integration occurs at multiple sites within a single specific locus (AAVS1) in 60–70% of cases, which maps to human chromosome 19q13.3-qter. AAV vectors in which the *rep* gene is deleted probably integrate randomly, suggesting that *rep* gene products (Rep78 and Rep68) are important for this process (Kumar and Leffak, 1991; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997). Vectors based on AAV may offer considerable advantages with respect to toxicity and immune response because they are deleted for all virally encoded proteins. In some tissues, transduction appears to be limited by inefficient conversion of the incoming single-stranded DNA genome to a transcriptionally active double-stranded template (Fisher *et al.*, 1996; Ferrari *et al.*, 1996). This process can be facilitated by co-infection with adenovirus through expression of the early gene product E4 orf6 or herpes viruses and some of their derivative vectors (unpublished results). Similar increases in transduction efficiency have been reported after treatment of cultured cells with γ-irradiation and topoisomerase inhibitors, and more recently after γ-irradiation *in vivo* (Alexander *et al.*, 1994; Russell *et al.*, 1995; Koeberl *et al.*, 1997). The mechanism of this process is undetermined, but may involve upregulation of DNA repair enzyme activity.

We have previously shown that rAAV vectors encoding the reporter gene *LacZ* efficiently transduce the RPE and are more efficient at transducing the neuroretina than AV vectors (Ali *et al.*, 1996). This is consistent with recent data that support the utility of this vector system for transduction of post-mitotic neuronal and muscle cells (Kapplitt *et al.*, 1994; Xiao *et al.*, 1996; Kessler *et al.*, 1996; Zolotukhin *et al.*, 1996; Fisher *et al.*, 1997). We now extend our original observations, and show that levels of transduction increase over a period of time, and that this process can be augmented *in vivo* by sublethal doses of γ-irradiation.

MATERIALS AND METHODS

Vectors and production of recombinant viruses

pTRCMVβ (gift from N. Muzyczka) consists of a *LacZ* reporter gene, cytomegalovirus (CMV) immediate-early promoter-enhancer, late gene 16S/19S splice donor/splice acceptor signal, and a heterologous polyadenylation signal. This cassette is flanked by 165-bp terminal repeat sequences derived from the wild-type AAV genome. pcp5RepCap was constructed by cloning a *Bal* I fragment containing both *rep* and *cap* genes into pcDNA3 (Invitrogen) deleted for the CMV promoter. The SV40 origin in this vector permits episomal replication in cell lines expressing the SV40 large T antigen. Recombinant viral particles were produced as previously published (Chiorini, 1995). Twenty confluent 150-mm plates of COS-7 (ATCC CRL1651) cells were trypsinized and suspended at a cell concentration of 2×10^7/ml in 100% RPMI, 20% heat-inactivated fetal calf serum (FCS). Five hundred microliters of cell suspension was placed in an electroporation cuvette (Biorad) with 30 μg of pTRCMVβ and 30 μg pcRepCap and incubated on ice for 10 min. Cells were mixed gently, pulsed in a BioRad gene pulser (960 μF, 250 V), and returned to ice for a further 10 min. Contents of all cuvettes were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and antibiotics, and replated in ten 150-mm dishes. Dead cells were removed the next day, and after a further 24 hr, wtAd5 was added to the plates in a total volume of 6 ml of DMEM at a multiplicity of infection (moi) of 5–10). Forty-eight hours later, at completion of the cytopathic process, cells were harvested and pelleted by centrifugation. The pellet was resuspended in TD buffer (140 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* K_2HPO_4, 25 m*M* Tris HCl pH 7.4), and lysed with 500 μl of 0.25% trypsin and 500 μl of 10% sodium deoxycholate per 7.5 ml of TD suspension. AAV vector stocks were purified from sequential caesium chloride gradients, dialyzed against HEPES-buffered saline, concentrated by ultrafiltration (Microcon 30), and heated to 56°C for 20 min to inactivate residual adenovirus. Infectious titer was determined by co-infection of HeLa cells with wtAd5 at a moi of 5. Purity of viral stocks was confirmed by electron microscopy and by absence of cytopathic effect on HeLa cells.

Intraocular injections

Mice were anesthetized by intraperitoneal (i.p.) injection of 0.2 ml Hypnorm (Janssen Pharmaceutical Ltd, Oxford), and Hypnovel (Roche, Welwyn Garden City) mixed 1:1:6 with distilled water. Following dilation with 1% Tropicamide (1% Mydriacyl, Alcon Labs, Watford), the eyes were protruded by gentle pressure on the animal's mandible. Once the eye was

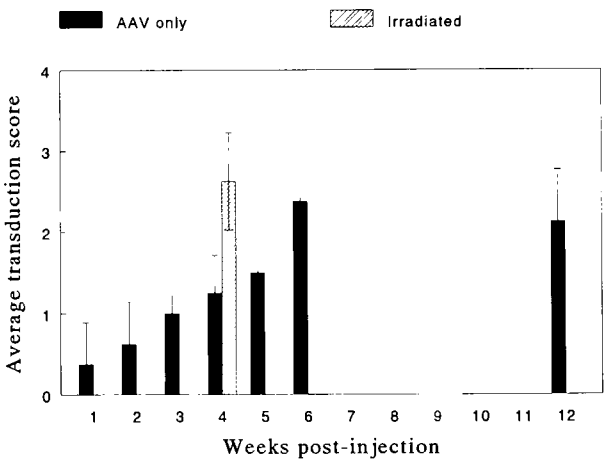

FIG. 1. AAV-mediated transduction after subretinal injection increases with time and is enhanced by γ-irradiation. Transduction efficiency of AAV following subretinal injection was scored after X-Gal incubation of the whole eye cup and examination under a dissecting microscope. The scores were assigned on the following basis: a score of 0 was given to eyes in which no blue staining was observed; a score of 1 was given to eyes in which there was a minimal staining; a score of 2 was given to eyes with a medium elvel of staining; and a score of 3 given to eyes with the most staining. The effects of sublethal irradiation on transduction efficiency 4 weeks after injection is also shown. Data from 46 animals (92 eyes) is shown: 12 eyes were analyzed at each point except at 12 weeks, when 8 eyes were scored. Error bars represent SD.

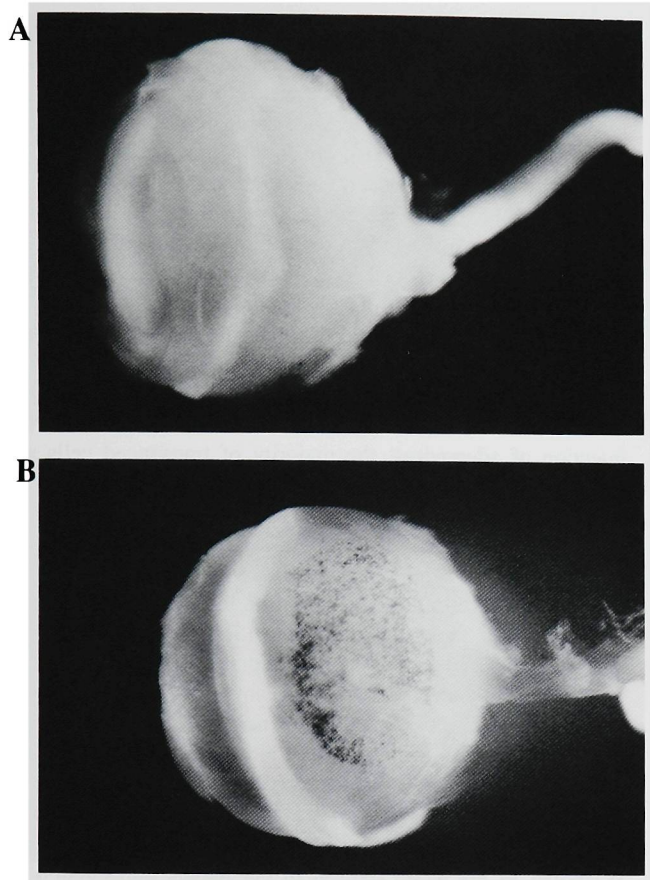

FIG. 2. Macroscopic photographs showing eyes taken from BALB/c mice at 2 weeks (A) and 6 weeks (B) following subretinal injection of AAV.LacZ. The eyes were prefixed in paraformaldehyde and the cornea, iris, and lens removed before overnight incubation in X-Gal. The density of blue staining increases substantially over time.

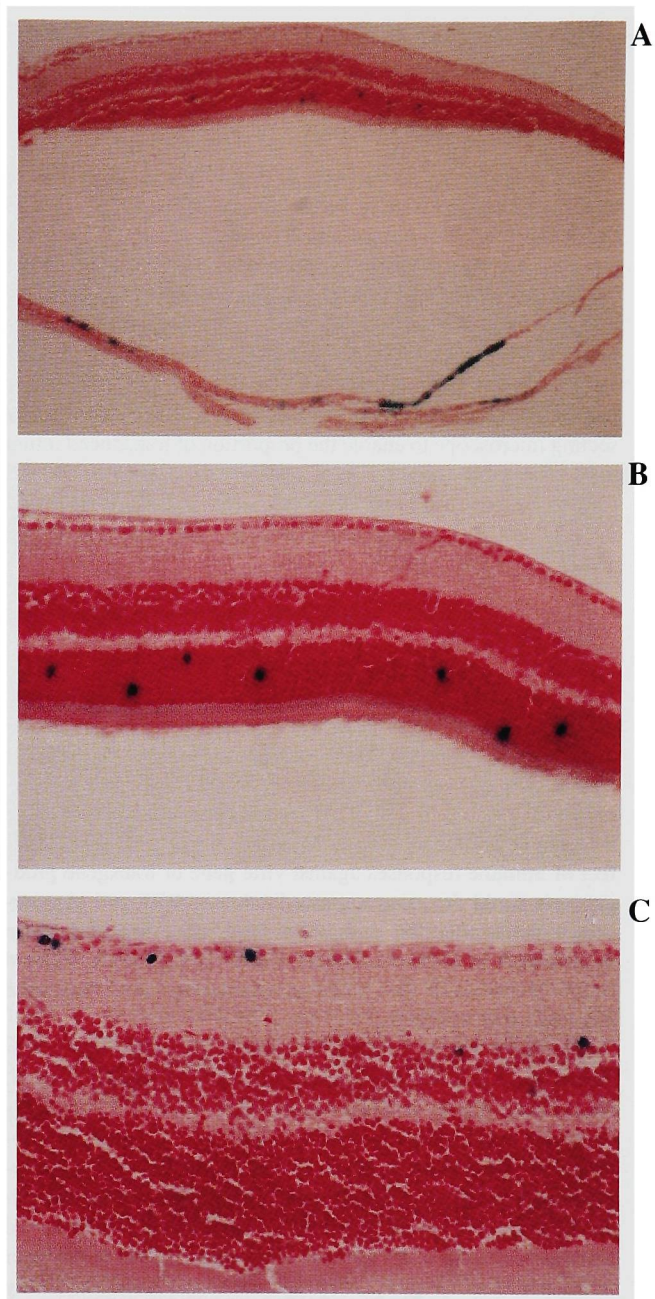

FIG. 3. A and B. Wax sections of 10 μm through retinas 6 weeks after subretinal injection reveal transduced RPE cells as well as transduced photoreceptor cells (counterstained with Nuclear Fast Red; 25× and 50× objectives). C. A 6-μm wax section through the retina, 6 weeks after intravitreal injection, showing transduced ganglion cells as well as an occasional transduced cell in the inner nuclear layer (counterstained with Nuclear Fast Red, 50× objective).

proptosed, it was held in this position by a rubber sleeve that was placed around the eye with a pair of forceps. The procedure is best described by analogy with a button hole, in which the rubber sleeve is the button hole and the mouse eye the button. It was held in position by the sleeve, which has a slit in the center to fit the eye. The pressure of the rubber sleeve on the eye was always moderate and did not block the circulation. Using this technique made fixation with ocular muscle sutures unnecessary.

Subsequently, the eye was covered with 2% hypromellose (methylcellulose) in saline and a small cover slip was fitted. This allowed surgery to be performed under direct retinoscopy thorough an operating microscope. The tip of a 1.5-cm, 34-gauge hypodermic needle (Hamilton, Switz) was guided in between the coverslip and the rubber sleeve to the sclera of the mouse eye and then injected tangentially through it causing a self-sealing wound tunnel. The needle tip was brought into focus between the retina and RPE and approximately 2 μl of viral stock was injected to produce a retinal detachment. For injection into the vitreous, the eye was protruded using a rubber sleeve and additionally stabilized by holding an extraocular muscle with a pair of fine forceps. The 34-gauge needle was injected through the cornea and iris in close proximity to the limbus of the mouse eye. To avoid the large lens, the needle was guided parallel to the sclera before being directed into the vitreous behind the lens. Two microliters of viral suspension was injected once the needle tip could be seen in the vitreous cavity behind the lens. All of the mice were BALB/c (Harlan Olac Ltd., Bicester). A total of 54 animals underwent this procedure and they were 4–8 weeks old at time of injection. Six

mice received single-dose sublethal γ-irradiation (5 Gy, Gammacell 1000) 24 hr after injection.

X-Gal staining and histology

Animals were killed by cervical dislocation and the enucleated eyes were prefixed by immersion in 10% buffered formalin for 45 min. The cornea and lens were separated from the eye cups and then all were rinsed in phosphate-buffered saline (PBS) and incubated overnight at room temperature with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Calbiochem, La Jolla, CA) in a solution containing 10 m*M* $K_3Fe(CN)_6$, 10 m*M* $K_4Fe(CN)_6$, 2 m*M* $MgCl_2$, in PBS. After incubation with X-Gal, the eyes were examined under a dissecting microscope to enable the proportion of transduced retina to be scored. The tissue was then fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 5–10 μm. Sections were counterstained with Nuclear Fast Red and examined by light microscopy.

RESULTS AND DISCUSSION

Previously, we have demonstrated that after subretinal injection, AAV is up to 2,000-fold more efficient than AV at transducing photoreceptor cells (Ali *et al.*, 1996). In these experiments, we consistently failed to see gene expression at early time points after injection. Using nude mice to eliminate the effect of immune responses against viral gene or transgene products, we could demonstrate transduction of RPE and photoreceptor cells after 28 days. We have now repeated these experiments in immunocompetent mice using highly purified rAAV preparations free from contaminating adenovirus (see Materials and Methods).

The AAV vector consists of a CMV immediate-early promoter, and *LacZ* reporter gene (pTRCMVβ, obtained from N. Muzyczka). Highly purified recombinant AAV (10^6 infectious particles) was injected in a total volume of 2 μl either by subretinal or intravitreal injection into 4- to 8-week-old immunocompetent BALB/c mice. At weekly time points after subretinal injection, mice were sacrificed and enucleated. We observed a gradual increase in the number of transduced cells in the outer retina over time (Fig. 1). Although there was some variability in levels of transduction between different animals, which may reflect the difficulty of precise injections, or variable degrees of tissue damage incurred during this process, there was clearly much greater transduction after 6 weeks than after 2 weeks post-injection (Fig. 2A,B). Eight eyes from 4 animals were still positive 3 months after subretinal injection (data not shown). We found no evidence of inflammatory cell infiltration at any time point examined. After evaluation under the dissecting microscope, eyes were embedded in paraffin wax and sectioned. Transduced cells consisted mostly of RPE cells with scattered photoreceptors (Fig. 3A,B). Two weeks after injection, up to 5% of RPE cells were transduced in approximately one-quarter of the retina—an area corresponding to the retinal detachment. No transduced photoreceptors were observed at this time point. After 6 weeks, up to 1% of photoreceptors and 90% of RPE cells were transduced in the area of detachment. The occasional transduced cell was observed in the inner nuclear layer (data not shown). Intravitreal injection of AAV resulted in diffuse transduction, mostly of the inner retina (Fig. 4A,B), which also increased with time. Here we compared transduction levels 2 weeks and 6 weeks post injection. Upon sectioning, it was apparent that the transduced cells were mostly ganglion cells (Fig. 3C). Up to 5% of the ganglion cells throughout the retina were transduced 6 weeks after injection. We also observed the occasional transduced cell in the inner nuclear layer (Fig. 3C) and the occasional transduced photoreceptor. The pattern of transduction after intravitreal injection of AAV is quite different from that seen after intravitreal injection of adenovirus. Following intravitreal injection of AAV, almost all of the transduced cells are in the inner retina, whereas after intravitreal injection of adenovirus, the majority of transduced cells are found in the non-neural cells of the anterior segment (see Introduction). The reasons for this are not clear.

Transduction by AAV vectors has been shown to be facilitated by administration of DNA-damaging agents both *in vitro* and *in vivo*. Eight mice were therefore injected after receiving sublethal whole-body γ-irradiation (5 Gy) from a cesium source. Compared with four nonirradiated controls, levels of transduction at 4 weeks were significantly enhanced (Fig. 1 and 5A,B). Although there was more uniform transduction, the treatment did not alter the proportion of transduced photoreceptor cells relative to transduced RPE.

Inherited diseases of the retina and the RPE are good candidates for therapies based on gene transfer because of the accessibility of the target tissues. In our hands, transduction of photoreceptor cells by adenoviral vectors has been inefficient, associated with toxicity at high moi, and with immunogenicity against proteins expressed from the vector backbone. These latest studies reinforce our previous observations that AAV vectors may have utility for therapeutic gene transfer to neuroretinal cells. The increase in transduction over time mirrors previous studies *in vitro* and *in vivo*, and suggests that host cell mechanisms are able to convert incoming vector genomes to transcriptionally active templates in the absence of co-infecting helper virus. We have also shown that prior administration of sublethal doses of γ-irradiation facilitates this process, although it is unclear whether this reflects induction of host cell repair polymerase activity or facilitated recruitment to the nuclear transcriptional machinery (Ferrari *et al.*, 1996; Fisher et al., 1996; Weitzman *et al.*, 1996). It is unlikely that the immunosuppressive effects of the treatment were responsible for the enhanced expression because we could eventually detect high-level expression after several months in nonirradiated immunocompetent animals. Tissue damage incurred at the site of subretinal injection could also enhance transduction, although the efficacy of intravitreal injection suggests that this is not a prerequisite for gene expression.

Removal of all virus-encoded genes from the vector backbone is beneficial to the packaging capacity of the virus, and possibly to its immunogenicity, but may be detrimental to the process by which wild-type AAV establishes latent infection. It is likely that vectors deleted for AAV *rep* integrate randomly. Although we have not examined the frequency of integration of the vector genome, recent studies in which rAAV particles were injected into post-mitotic muscle tissue demonstrate the

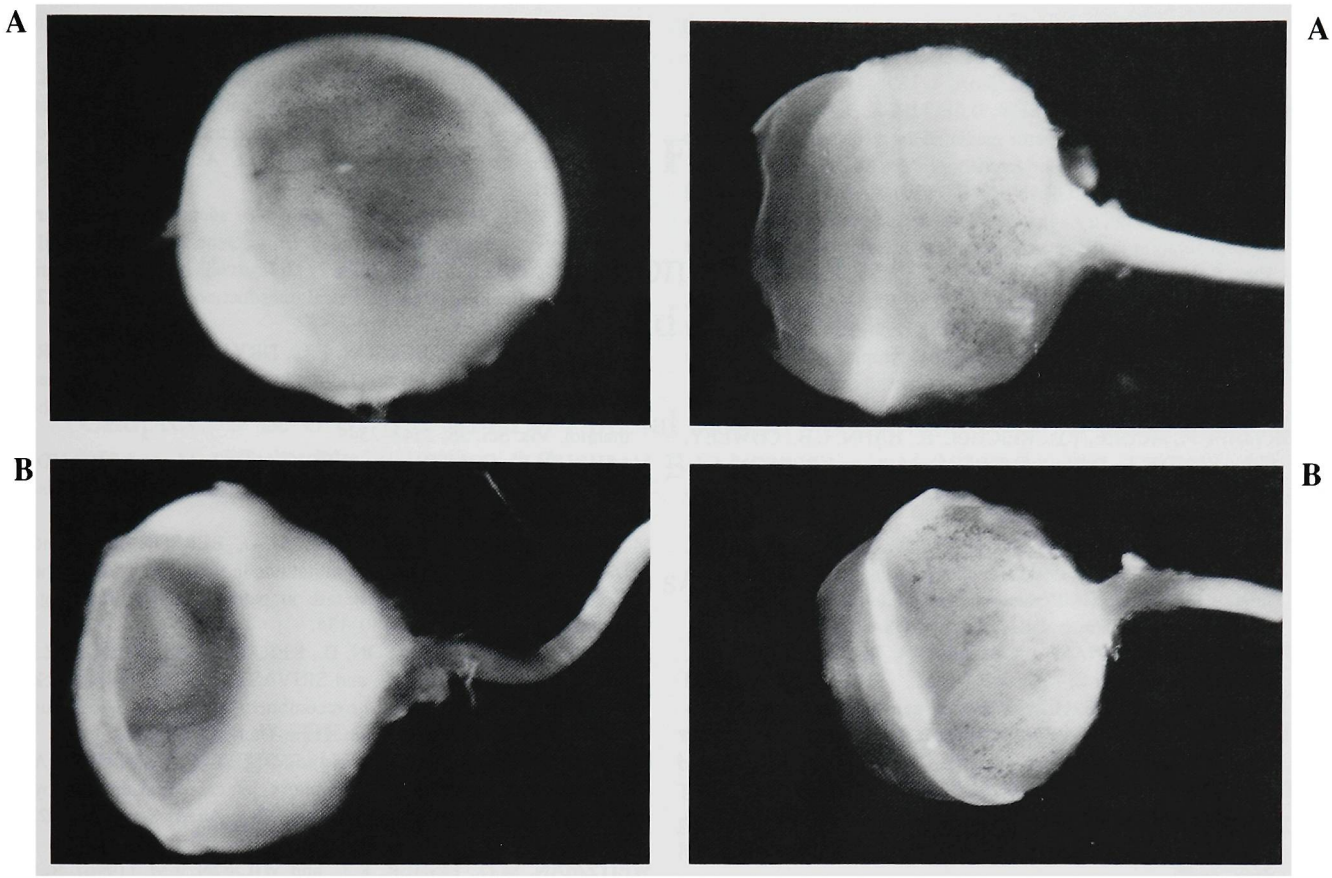

FIG. 4. A and B. Macroscopic photographs showing punctate X-Gal-positive staining in the posterior eye cups taken from BALB/c mice at 6 weeks following intravitreal injection of AAV.LacZ. The eyes were prefixed in paraformaldehyde and the cornea, iris, and lens removed before overnight incubation in X-Gal.

FIG. 5. Transduction of the retina by AAV is substantially enhanced by whole-body irradiation. The macroscopic photographs show eyes taken from BALB/c mice at 4 weeks following subretinal injection of AAV.LacZ. The eyes were prefixed in paraformaldehyde and the cornea, iris, and lens removed before overnight incubation in X-Gal. A shows an eye from a nonirradiated control animal. B shows an eye from mouse that received sublethal γ-irradiation (5 Gy) 24 hr after injection.

formation of head-to-tail or tail-to-tail tandem arrays consistent with genomic integration (Xiao *et al.*, 1996; Fisher *et al.*, 1997). A similar mechanism may, in part, explain the stability of photoreceptor transduction seen in this study and is currently under investigation. Particularly impressive is the lack of an immune response to the transduced cells, which is in contrast to our experience using first-generation E1-deleted adenoviral vectors. Paucity of immune responses against the vector and reporter transgene in the context of AAV probably contribute to the stability of transduction.

Future studies will focus on the administration of therapeutic genes to murine models of RP. However, the rapidity with which irreversible retinal degeneration becomes established in these models may require the kinetics of transduction by AAV vectors to be facilitated. This process is now under investigation. Although γ-irradiation accelerates AAV-mediated gene transduction in murine models, human retinal degeneration is progressive over long periods of time so that some delay in gene expression is unlikely to prevent therapeutic efficacy.

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